PCT

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 15/19, C12P 21/02 C07K 13/00, C12N 1/21	A2	(11) International Publication Number:	WO 91/11515
		(43) International Publication Date:	8 August 1991 (08.08.91)

(54) Title: LEUKOCYTE-DERIVED GROWTH FACTOR

(57) Abstract

A gene encoding a leukocyte-derived growth factor (LDGF) has been isolated, cloned and sequenced. LDGF is believed to correspond to a PDGF-like monocyte-derived growth factor with chemotactic activity which is found in human wound fluid. Protease-resistant and other analogues of LDGF, as well as recombinant LDGF of native amino acid sequence, may now be produced by gene expression in transformed hosts.

```
                          G AGG CAA CTC ACC CTC ACT CAG ACG TCT TCT GGT  34

TCT GGA AAC AAC TCT AGC TCA GCC TTC TCC ACC ATG AGC CTC AGA  79
                                            Met Ser Leu Arg

CTT GAT ACC ACC CCT TCC TGT AAC AGT GCG AGA CCA CTT CAT GCC  124
Leu Asp Thr Thr Pro Ser Cys Asn Ser Ala Arg Pro Leu His Ala

TTG CAG GTG CTG CTG CTT CTG TCA TTG CTG CTG ACT GCT CTG GCT  169
Leu Gln Val Leu Leu Leu Leu Ser Leu Leu Leu Thr Ala Leu Ala

TCC TCC ACC AAA GGA CAA ACT AAG AGA AAC TTG GCC AAA GGC AAA  214
Ser Ser Thr Lys Gly Gln Thr Lys Arg Asn Leu Ala Lys Gly Lys
ΔPlatelet Basic Protein            ΔLAPF-4/CTAPIII  ΔBTG

GAG GAA AGT CTA GAC AGT GAC TTG TAT GCT GAA CTC CGC TGC ATG  259
Glu Glu Ser Leu Asp Ser Asp Leu Tyr Ala Glu Leu Arg Cys Met

TGT ATA AAG ACA ACC TCT GGA ATT CAT CCC AAA AAC ATC CAA AGT  304
Cys Ile Lys Thr Thr Ser Gly Ile His Pro Lys Asn Ile Gln Ser

TTG GAA GTG ATC GGG AAA GGA ACC CAT TGC AAC CAA GTC GAA GTG  349
Leu Glu Val Ile Gly Lys Gly Thr His Cys Asn Gln Val Glu Val

ATA GCC ACA CTC AAG GAT GGG AGG AAA ATC TGC CTG GAC CCA GAT  394
Ile Ala Thr Leu Lys Asp Gly Arg Lys Ile Cys Leu Asp Pro Asp

GCT CCC AGA ATC AAG AAA ATT GTA CAG AAA AAA TTG GCA GGT GAT  439
Ala Pro Arg Ile Lys Lys Ile Val Gln Lys Lys Leu Ala Gly Asp

GAA TCT GCT GAT TAA TTT GTT CTG TTT CTG CCA AAC TTC TTT AAC  484
Glu Ser Ala Asp***

TCC CAG GAA GGG TAG AAT TTT GAA ACC TTG ATT TTC TAG AGT TCT  529

CAT TTA TTC AGG ATA CCT ATT CTT ACT GTA TTA AAA TTT GGA TAT  574

GTG TTT CAT TCT GTC TCA AAA ATC ACA TTT TAT TCT GAG GAA GGT  619

TGG TTA AAA GAT GGC AGA AAG AAG ATG AAA ATA AAT AAG CCT GGT  664

TTC AAC CCC TC                                               675
```

LEUKOCYTE-DERIVED GROWTH FACTOR

BACKGROUND OF THE INVENTION
Field of the Invention

This invention relates to a leukocyte-derived
growth factor which has PDGF-like activity but is
structurally distinct from PDGF. This novel growth factor
is useful in the healing of wounds. Antibodies against this
growth factor are useful in the treatment of fibrotic
disorders.

Information Disclosure Statement

Within minutes of an injury, platelets adhere to
the wound site and aid in clot formation. The phagocytic
cells (leukocytes and macrophages) then debride the wound,
followed by the connective tissue cells (fibroblasts and
smooth muscle-like cells) which proliferate and deposit
extracellular matrix. Finally, endothelial cells
revascularize the wound site.

Chemotaxis is the directed migration of a cell
along a gradient toward the source of a chemical. A
chemoattractant is a chemical which specifically stimulates
chemotaxis. It is the sequential production of cell type-
specific chemoattractants which is responsible for the
ordered recruitment of phagocytes, fibroblasts and
endothelial cells to the wound site. C5A, platelet
factor 4, elastin peptides and certain synthetic N-
formylmethionyl peptides attract phagocytes (neutrophils and
monocytes). Fibronectin and platelet-derived growth factor
summon matrix producing cells. Fibronectin also stimulates
endothelial cell migration.

· 2

The ability of a cell to respond to a particular
factor acting as a chemoattractant is dependent on several
biochemical events. First, the attractant molecules must be
produced at a site from which they can diffuse into the
5   surrounding tissues and thereby reach the cell in question.
Second, the target cell must possess a specific means of
detecting small quantities of the chemoattractant (i.e.,
specific high affinity receptors). Finally, the occupancy
of the receptor by the chemoattractant must initate
10   biochemical changes within the cell which activate the
cytoskeletal machinery for cell movement.

The proliferation of connective tissues at the
wound site is promoted by various polypeptide growth
factors. Competence factors (PDGF, fibroblast growth
15   factor, monocyte-derived growth factor) activate quiescent
cells in the G_o phase of the cell cycle, enabling them to
respond to progression factors. The latter (insulin,
somatomedin A or C, alveolar macrophage-derived growth
factor) stimulate the cells to enter the S-phase.

20   PDGF is both a connective cell chemoattractant and
a mitogen for such cells, leading to it being termed a
"mitoattractant". However, there are other growth factors
which have mitogenic action on fibroblasts but are not
chemoattractants for them (e.g., epidermal growth factor,
25   transforming growth factors α and β, somatomedins A and C,
and insulin). EGF is a mitoattractant for intestinal
epithelial cells.

The extracellular matrix is composed of collagens,
glycoproteins and proteoglycans. Cells do not bind directly
30   to collagen but interact via glycoproteins called attachment
factors. Such factors include fibronectin, laminin and
chondronectin.

Fibrosis is the excessive deposition of connective
tissue, largely collagen, in parenchymal tissue, and may be

considered "the dark side of wound repair" as it is believed
to be the result of an overly prolonged signal for
connective tissue repair.

5   See generally Ross, et al., The Biology of
Platelet-Derived Growth Factor, Cell, 46:155-169 (1986);
Ross, Platelet-Derived Growth Factor, Ann. Rev. Med., 38:71-
79 (1987); Grotendorst, et al., Production of Growth Factors
(PDGF & TGFß) at the Site of Tissue Repair, in GROWTH
FACTORS AND OTHER ASPECTS OF WOUND HEALING: BIOLOGICAL AND
10   CLINICAL IMPLICATIONS, pp. 47-54 (1988); Grotendorst, et
al., Molecular Mediators of Tissue Repair, in Soft and Hard
Tissue Repair; Biological and Clinical Aspects, pp. 20-40
(1984); Grotendorst and Martin, Cell Movements in Wound-
Healing and Fibrosis, Rheumatol., 10:385-403 (1986); and
15   Grotendorst, et al., Chemoattractants in Fibrotic Disorders,
In Fibrosis, Ciba Foundation Symposium (1985).

        Human platelet derived growth factor is a dimeric
glycoprotein with a molecular weight of about 30,000
daltons. Nanomolar concentrations of PDGF stimulate
20   replication in 3T3 cells. Reduction of its disulfide bonds
destroys PDGF's mitogenic activity. The A- and B- chains of
PDGF are related to each other; it is not known whether PDGF
is a heterodimer or a mixture of homodimers. The A chain is
also heterogeneous, existing in 18kD, 15kD, 14kD and 11kD
25   forms, while the B-chain's only major form is 16kD. See
Johnsson, et al., Biochem. Biophys. Res. Comm., 104:66
(1982). PDGF has been purified to homogeneity, see Heldin,
et al., Biochem. J., 193:907 (1981). The cDNA sequence of
the PDGF A-chain gene is given in Betsholtz, et al., Nature,
30   320:695 (1986). The amino acid sequence of both PDGF-1 and
PDGF-2 may be found in Doolittle, et al., Science, 221:275
(1983). For expression of PDGF analogues, see Zymogenetics,
EP Appln 177,957 (1986). Anti-PDGF antibodies are
available, as are antibodies against synthetic peptides

4

derived from the A or B chains (e.g., vs. A92-119 or B79-107). The biological activity of PDGF is reviewed in Ross, Ann. Rev. Med., 38:71 (1987) and Ross, et al., Cell, 46:155 (1986).

5   There are several aspects of the platelet derived growth factor molecule which may be disadvantageous to its use in the acceleration of wound repair. First, PDGF is a dimeric glycosylated protein which will be more difficult and expensive to produce than smaller non-glycosylated

10   monomeric peptides. Also, there are three isoforms of PDGF which exist (AA, AB, BB). These isoforms exhibit differential biological activity on connective tissue cells and it is not known at present what types and amounts of these are present at sites of tissue repair. However,

15   studies in our laboratory have shown the authentic PDGF peptides are present in only trace amounts in human wound fluid.

       An activity of particular interest is PDGF's chemoattractive activity. Grotendorst, Cell, 36:279-85

20   (1984) observed that cells responsive to PDGF as a mitogen (bovine aortic smooth-muscle cells, human skin fibroblasts, NIH/3T3 cells and NRK cells) also evinced a chemotactic response, while cells for which PDGF was not mitogenic (bovine aortic endothelial cells, MDCK cells, TERA cells,

25   and PAM 212 cells) were not chemoattracted, either. Grotendorst, et al., PNAS (USA), 78:3669 (1981) suggested that the migration of smooth muscle cells from the media to the intima of a blood vessel, which leads to the formation of an atherosclerotic plague may be PDGF-induced.

30   Connective Tissue Activating Peptide III (CTAP-III), also known as Low Affinity Platelet Factor-4 (LAPF4), is an acid-ethanol stable, slightly basic (isoelectric point 8.5) protein with a molecular weight of about 9,300 daltons, and composed of a single polypeptide chain of 85 residues

with a conformation influenced by intrachain disulfide
bonds.   Castor, et al., Arthritis and Rheumatism, 20:859
(1971); Castor, et al., PNAS (USA), 80:765 (1983); Castor,
et al., Biochemistry, 24:1762 (1985); Castor, J. Rheumatol.,
Suppl. 11:55 (1983).   Its amino acid sequence is known, see
Castor, et al. (1983), and anti-CTAP III antibodies are
available, see Castor (1983).   Its biological activities
(seen at microgram levels) reportedly include promotion of
DNA synthesis, hyaluronic acid synthesis, sulfate
incorporation into proteoglycans, prostaglandin E_2
synthesis, plasminogen activator secretion, glucose uptake,
and lactate formation, see Castor, et al. (1985).
Mullenbach, et al., J. Biol. Chem., 261:719 (1986)
synthesized a gene which codes for CTAP-III and expressed
this protein in yeast.   The HPLC-purified recombinant
protein was said to exhibit half-maximal mitogenic
(stimulation of DNA synthesis) activity at
$10^{-7}M$ levels.

        The mitogenic activity of CTAP-III (LAPF-4) has
been questioned by Holt, et al., Biochemistry, 25:1988
(1986).   Holt believes that while the first crude
preparations of LA-PF4 tested were mitogenic for 3T3 cells,
his more highly purified LA-PF4 was inactive.   He remarked,
"the purity of the tested material is a crucial issue".

        Beta-thromboglobulin (β-TG) is a mitogenically
inactive truncated form of CTAP-III.   It is missing the N-
terminal tetrapeptide of CTAP-III; the remaining 81 residues
apparently are not independently active as mitogens.   β-TG
may, however, be chemotactic.   See Holt, et al., Exp.
Hematol., 16:302 (1988).

        Platelet basic protein (PBP), on the other hand,
is CTAP-III with a nine residue N-terminal extension.   PBP
was initially reported to be a 3T3 cell mitogen at
concentrations of 1-10 ng/ml; see Paul, et al., Thrombosis

6

Research, 18:883 (1980). This group later recanted,
declaring that purified PBP was free of mitogenic activity.
See Holt, et al. (1986). We are not aware of any attempt to
express PBP by recombinant DNA techniques.

5   Shimokado, et al., Cell, 43:277 (1985)
fractionated medium conditioned by alveolar macrophages,
obtaining fractions with mitogenic activity. An anti-PDGF
antibody immunoprecipated a monomeric protein of a molecular
weight of 12-13,000 daltons. The protein was not further
10   characterized or purified.

Martinet, et al., Nature, 319:158 (1986) reported
that activated human blood monocytes (macrophages) release a
substance possessing PDGF-like activity, specifically,
chemotactic activity for mesenchymal cells and growth-
15   competence activity for fibroblasts. The substance
displaced PDGF from its natural fibroblast receptors and
from anti-PDGF antibody. Like PDGF, Martinet's mediator
appeared to require disulfide bonding for biological
activity.

20   Pencev and Grotendorst, Oncogene Research, 3:333
(1988 issue; actually published in February, 1989) and
Matsuoka and Grotendorst, PNAS (USA), 86:4416 (June 1989)
relates to work by the inventors, relating to a chemotactic
growth factor. Their work was confined to study of the
25   partially purified native protein.

Collagen, EP Appln 243,179 describes a wound
healing composition comprising a growth, chemotactic or
differentiation factor.

Urry, U.S. 4,693,718, relates to the stimulation
30   of fibroblast chemotaxis by synthetic peptides corresponding
to peptide repeats in tropoelastin.

No admission is made that any reference cited
herein is prior art or pertinent prior art. The dates given
are the nominal dates appearing in the paper and may not

correspond with the publication dates for patent purposes. All references are incorporated by reference to the extent pertinent.

SUMMARY OF THE INVENTION

5   PDGF is not the principal mitoattractant in human wound fluid. While the fluid reacted with polyclonal anti-human PDGF antibody, the reactive peptides did not correspond well with either PDGF or its subunits. PDGF has a molecular weight of about 30kDa; the two species of this invention
10   were 16-17kDa and 34-36kDa, respectively, and did not comigrate with PDGF. Immunopurified peptides were not recognized by PDGF A- or B- chain specific antisera.

       The 16kDa peptide is believed to correspond with a 16kDa monomeric chemoattractant secreted by human monocytes
15   and also found to be distinct from PDGF A or B.

       Using an anti-PDGF polyclonal antibody as a probe, a cDNA of interest was identified in a cDNA library derived from activated human peripheral blood monocytes. This cDNA encoded a protein which may be described as PBP with a 34
20   amino acid N-terminal extension. This new peptide is referred to herein as "Leukocyte Derived Growth Factor", (LDGF) since it is secreted by neutrophils as well as monocytes.

       This cDNA may be operably linked to a promoter
25   functional in a selected host so that LDGF may be expressed in that host.

       The native LDGF acts in a manner which is indistinguishable from that of platelet derived growth factor (PDGF). That is, LDGF is a potent chemotactic and
30   mitogenic factor for connective tissue cells such as fibroblasts, smooth muscle cells and astroglial cells, but not for endothelial cells, epithelial cells or leukocytes. Experimental data suggest that this is due to the interaction of both PDGF and LDGF with the same cell surface

8

receptor molecules. These receptors do not bind other known growth factors including transforming growth factor alpha or beta, epidermal growth factor, insulin-like growth factors, fibroblast growth factors, interleukins, tumor necrosis

5   factor, interferons, or other members of the gro-gene family that have been tested including Platelet factor 4, connective tissue activating peptide III, platelet basic protein, and melanoma growth stimulating activity (gro gene product).

10   LDGF, and analogues thereof, may be used to facilitate wound healing. In particular, they will be useful in the treatment of many healing impaired conditions including chronic skin ulcers such as decubitis, venous stasis and diabetic ulcers where we find increased levels of

15   this factor compared to normal healing wounds. It would also be useful for the treatment of poor wound healing in surgical wounds where the patients have undergone treatment which impairs healing such as chemotherapy with antineoplas- tic drugs or steroids. In addition to skin wounds this

20   material would be useful for the treatment of other injuries and surgical procedures where an acceleration of connective tissue formation is desired including bone grafts, bone fracture non-unions, artificial joint replacements, and tendon repairs as we have shown that it is normally present

25   at these sites as well. In the ophthalmic area this material could be very useful for the acceleration of surgical wounds after anterior segment surgery where little bleeding occurs and the wounds heal poorly. Because the material acts in a manner indistinguishable from PDGF and we

30   have demonstrated the efficacy of PDGF in most of the above situations we are certain that this material will provide a suitable stimulation to the same target cells as PDGF acts on.

Because it is smaller than PDGF, contains only a single subunit, and lacks any glycosylation sites so that it is not a glycoprotein as is PDGF, LDGF will be much easier and less expensive to manufacture.

Studies discussed herein have indicated that LDGF is the principal growth factor present in human wounds during the early stages of the repair process. Therefore, addition of this factor more closely mimicks the natural process of wound repair. Use of this factor is less likely to cause unwanted side effects compared to PDGF which is present in much lower amounts at the wound sites.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 presents the cDNA and translated amino acid sequence for LDGF. Platelet-basic protein, LAPF-4/CTAP-III and ß-TG have different N-terminals and a common C-terminal relative to LDGF. The N-termini of these related proteins are marked.

Figure 2 maps the hydrophilic and hydrophobic regions of LDGF by the methods of (a) Kyle and Doolittle, window 7, and (b) Hopp and Woods, window 6. Hydrophilic regions are more likely to lie on the surface and thereby be antigenic.

Figure 3 surveys the (a) alpha helix, (b) beta sheet and (c) reverse turn potential across the LDGF sequence, using the algorithm of Chou and Fasman.

Figure 4 shows the net charge of LDGF for each window of 10 residues.

Figure 5 points out amphipathic alpha-helixes (window 11, degrees 100), which are potential T cell determinants.

Figure 6 shows PDGF-like chemotactic activity in media and cell extracts of non-activated and LPS-activated human peripheral blood monocytes.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention relates to a leukocyte-
derived growth factor of value in the treatment of wounds.
It is believed to induce the chemotaxic movement of
5 connective tissue cells to the wound site and the subsequent
proliferation of those cells.

In one aspect, the present invention relates to
the purification of a naturally occurring chemotactic and
mitogenic factor from media conditioned by activated
10 peripheral blood monocytes or human wound fluid. This
factor is characterized as a 16 kD manomeric protein which
is recognized by anti-PDGF antibody but not by PDGF A chain-
or B chain-specific antibodies, which interacts with the
cellular receptor for PDGF, and which differs from PDGF A
15 and B chains in its sensitivity to cyanogen bromide and
formic acid. In a preferred embodiment, this factor is
partially purified by affinity chromatography on immobilized
heparin.

In a second aspect, this invention relates to the
20 discovery that a gene encoding a novel chemotactic factor
may be identified by immunoscreening an expression library
with anti-PDGF antibodies. This gene or fragments thereof
may be used as an oligonucleotide probe to identify and
isolate related genes.

25 In a third aspect, the invention contemplates the
cloning and expression of a gene encoding the novel factor
LDGF, whose amino acid sequence is given herein and which is
believed to be the chemotactic factor purified from
monocyte-conditioned media and human wound fluids.
30 Production of LDGF by recombinant techniques is preferred.

In a fourth aspect, the invention relates to the
development of novel chemotactic and/or mitogenic factors
obtained by expression of a mutagenized LDGF gene or by

11

chemical or enzymatic treatment of the recombinant LDGF
protein.

In a fifth aspect, the invention encompasses the
preparation of antibodies to LDGF-like proteins, and of the
corresponding anti-antibodies which may mimic certain of the
properties of LDGF.

LDGF may be obtained by purification from natural
sources, e.g., monocyte cell culture supernatants and human
wound fluids. However, it is more conveniently obtained
synthetically.

Preferably, LDGF is prepared by operably linking a
gene encoding the LDGF to a promoter, and expressing the
gene under the control of that promoter in a transformed
host cell. The gene may be a cDNA as in the Example, or it
may be a genomic LDGF sequence (identified using the LDGF-
related cDNA as a hybridization probe or anti-PDGF antibody
as an immunological probe against a genomic library), or it
may be a synthetic DNA (since the LDGF cDNA has been
sequenced).

The cell may be a prokaryotic (e.g., bacterial) or
a eukaryotic (e.g., yeast, mammalian) cell. The preferred
host cells are E. coli. The promoter must be functional in
the host cell; a favored promoter is T-7 phage. The LDGF is
preferably secreted by the transformed cell; if not, the
cell must eventually be lysed so that the LDGF may be
recovered.

The LDGF may then be purified to increase its
specific activity. Preferably, the LDGF is purified using
an anti-PDGF (or LDGF) immunoaffinity column and/or by
affinity chromatography on heparin-sepharose.

Purified LDGF may be used to raise polyclonal and
monoclonal antibodies against LDGF. These antibodies may be
used in the immunopurification of additional LDGF, in assays
for LDGF in wound fluid, or in the treatment of fibrotic

12

conditions.

Monitoring the level of LDGF in wound fluid might provide a useful indication of the healing activity at the wound site. The assay is preferably an immunoassay, and is conducted in a competitive or a sandwich format. In one competitive format, sample LDGF competes with labeled LDGF for an insolubilized antibody, which is preferably an anti-LDGF antibody but which could be an anti-PDGF antibody. In one sandwich format, sample LDGF is bound by both an insolubilized antibody and a labeled antibody. The order of introduction of the reactants may be varied.

Antibodies to LDGF may be used to block the LDGF/PDGF receptors and thereby treat fibrotic conditions resulting from overstimulation of the receptors.

While production by recombinant DNA techniques is preferred, the LDGF molecule (or analogues) may also be prepared by concatenation of amino acids or oligopeptides in vitro.

It is believed that analogues of LDGF may share its chemoattractive and mitogenic activity. We believe that the N-terminal extension (relative to PBP) of LDGF substantially enhances its activity and that the three cysteines of LDGF which are aligned with cysteines in the PDGF B chain protein are of special significance. Analogues may be prepared by site-specific mutagenesis (Zoller and Smith, 1984; DNA vol. 3, 479-488) of the LDGF gene and expression of the mutated gene. Preferably, the analogues are substantially homologous with at least the unique "N-terminal extension" of LDGF which distinguishes it from PBP.

It is this region of the LDGF molecule which adds significantly to the sequence homology with the PDGF-B chain including an additional cysteine residue. Also, hydropathy slots of this region of the molecule are very similar to the PDGF-B chain molecule providing further evidence of similar

three dimensional structures which would explain the
antibody and receptor cross-reactivity. Because we have
tested purified CTAP-III, PBP, and ß-TG in our biological
and immunological tests and found them to be inactive we
feel that the additional N-terminal peptide region is
essential for PDGF-like biological activity. The term
"LDGF-like protein" includes such analogues which
substantially contain the N-terminal extension but excludes
PDGF, PBP, CTAP-III and ß-thromboglobulin. Preferably, the
"LDGF-like protein" is more homologous with native LDGF as
defined herein than with PDGF, PBP, CTAP-III or ß-
thromboglobulin. Homology may be determined by the
algorithm of Needleman and Winsch, J. Mol. Biol. 48:443-453
(1970) with a window of 100, a gap penalty of 10, a size
penalty of 2 and a maximum gap of 50.

These analogues may differ from LDGF by single or
multiple insertions, deletions or substitutions. While it
is not possible to state with certainty, _a priori_, what the
effect of a given mutation will be, the following
generalizations are possible. A mutation is more likely to
affect activity if one or more of the following criteria is
satisfied:

    (1)   The mutation affects the 34-AA N-terminal
          extension which distinguishes LDGF from PBP.

    (2)   The mutation affects one of the amino acids
          which is conserved between LDGF and PDGF B
          chain.

    (3)   The mutation affects an amino acid which lies
          in a hydrophilic region of LDGF (see Fig. 2)
          and which therefore is likely to be exposed
          to solvent.

    (4)   The mutation affects an alpha helix or beta
          sheet region of LDGF as depicted by Fig. 3,
          particularly if the mutation substantially

14

alters the secondary structure tendency.

(5)   The mutation substantially increases or
        decreases the amphipathicity of an
        amphipathic alpha helix as identified by
        Fig. 5.

(6)   The mutation substantially alters the size of
        a residue lying in a hydrophobic region of
        LDGF and therefore thought to be a part of
        the core of the molecule.

(7)   The mutation replaces an amino acid with one
        .of a significantly different structure.

Schultz and Schirmer, <u>Principles of Protein
Structure</u> 14-16, 170 (1979) analyzed the frequency of amino
acid changes between corresponding proteins of homologous
organisms. This analysis reveals the existence of exchange
groups; amino acids within such a group exchange
preferentially with each other. Schultz and Schirmer
identify four such exchange groups:

    I   Phe, Tyr, Trp (aromatic amino acids)
    II   Lys, Arg, His (positive charged amino acids)
    III   Val, Leu, Ile, Met, Cys (large aliphatic
          amino acids)
    IV   Ser, Thr, Asp, Asn, Gly, Ala, Glu, Gln, Pro
          (small amino acids)

        Schultz and Schirmer also set forth the mutation
probabilities for each of the amino acids. <u>Id.</u>, 171-172.
The amino acids which are replaced most frequently are
serine, methionine, and asparagine; the least frequently
mutated amino acids are tryptophan, cysteine and tyrosine.
Tryptophan has the largest side chain. Cysteine can
participate in disulfide bridges. Tyrosine forms very
strong hydrogen bonds.

        A more stringent definition of conservative
substitions is adopted by the GENEPRO (Riverside Scientific)

15

alignment program, which recognizes the following groups:
(Ala, Gly), (Asp, Glu), (Phe, Tyr), (Ile, Leu, Val), (Lys,
Arg), (Asn, Gln), and (Ser, Thr).

5   If a person wishes to substantially alter the
biological activity of LDGF (e.g., to obtain LDGF with
increased affinity for its cell surface receptor, or to
change the balance of the chemotactic and mitogenic
activities of LDGF), mutations likely to affect activity
according to the above criteria will be introduced. Many of
10   these mutations will abolish rather than enhance activity,
of course. One strategy is to randomly mutagenize the LDGF
gene (either throughout its length, or focused on residues
likely to affect activity) and then screen for functional
mutants. Alternatively, candidate mutants may be prepared
15   individually by nonrandom site-specific mutagenesis and then
screened.

         For an overview of mutagenesis strategies see
Botstein and Shortle, Science, 229:1193 (1985). For focused
random mutagenesis in particular, see, e.g., Abarzua and
20   Marians, PNAS (USA), 81:2030-34 (1984); Fasano, et al., PNAS
(USA), 81:4008-12 (1984); Myers, et al., Science, 229:242
(1985); Matteuci and Heyneker, Nucleic Acids Res., 11:3113
(1983); and Wells, et al., Gene, 34:315-23 (1985).

         It may instead be the goal to make only
25   "conservative" mutations in LDGF, that is, changes unlikely
to affect activity. The aforementioned criteria are equally
useful here. Once again, where there is uncertainty as to
the effect of a change, focused random mutagenesis may be
employed.

30   Screening with anti-PDGF (or LDGF) antibodies may
also be useful, though immunological activity does not
necessarily correlate with biological activity. Analogues
may also be screened with PDGF (or LDGF) for ability to
competitively inhibit binding to the PDGF (or LDGF)

receptor.

It may be advantageous to prepare analogues of
LDGF which have increased stability to proteolytic
degradation. These analogues would have particular utility
5   in the treatment of chronic wounds where many experts feel
that an overproduction of proteases at the site of injury
underlies the basis for the impaired healing in these cases,
as the proteases degrade the natural growth factors present
at the wound site. These analogues would be created by
10   replacing some of the amino acids in the protease cleavage
sites which allow for the degradation of LDGF to PBP, CTAP-
III and beta-TG, which are inactive forms of the molecule.
For example, the two serine residues at position 35 and 36
could be replaced with threonine and the asparagine residue
15   at position 44 could be replaced with glutamine. (Both
conservative substitutions by GENEPRO criteria.) Other
suitable substitutions could be determined by focused random
mutagenesis at these and similar sites.

For techniques not described in detail here but
20   conventional in the molecular biology and immunology arts,
see Sambrock, Fritsch and Maniatis, Molecular Cloning: A
Laboratory Manual, Vols. 1-3 (Cold Spring Harbor: 2d ed.
1989), and Harlow and Lane, Antibodies: A Laboratory Manual
(Cold Spring Harbor: 1988).

25   **Example 1: Characterization of a Monocyte-Derived Growth
Factor Isolated from Conditioned Media**

MATERIALS AND METHODS

Growth Factors

Human PDGF was isolated from platelets and
30   purified to homogeneity by methods as described previously
(Grotendorst, 1984). PDGF A and B chain homodimers were
purchased from Biochem, Inc. or from AMGEN, Inc. Pure
material as determined by SDS PAGE gel electrophoresis and

reverse phase HPLC was used in all biological assays described below and to raise anti-human PDGF antibody.

Antibodies

Purified PDGF or synthetic peptides containing the amino acid terminal sequences of the PDGF A chain (amino acids 92-119 of the precursor molecule) and the PDGF B chain (amino acids 79-107) were used to raise specific antibodies in goats. Goats were immunized with 20 μg of purified PDGF or 50 μg of synthetic peptides in Freund's complete adjuvant by multiple intradermal injections. After the fourth rechallenge with 20 μg of pure PDGF (50 μg synthetic peptides) in Freund's incomplete adjuvant, immune sera were collected 7 days after the last immunization and tested for specificity by immunoprecipitation and Western blots. The tested immune sera did not show any cross reactivity with other isolated growth factors such as TGF-B (up to 50 ng of pure TGF-B in Western blot analysis) or EGF. Immune sera raised against the PDGF A (92-119) peptide and PDGF B (79-107) peptide proved to be sequence specific as determined by immuno dot blot and did not crossreact with peptides containing remaining amino acid sequences of the PDGF A or PDGF B chain molecule. The IgG fraction of the immune serum was isolated on DEAE Affi gel blue Sepharose (Biorad, Richmond, California, equilibrated with 0.02 NaCl, 0.02 Tris pH 8.0).

General Reagents

Lipopolysaccharide (LPS, E. coli 0127:B8) was purchased from Sigma Chemical Co., St. Louis, Missouri, FMet-Leu-Phe (MLP) from Peninsula Laboratories, San Carlos, California, and immune complexes from Cooper Biomedical, Malvern, Pennsylvania.

Preparation of LDGF from human monocytes and conditioned media

18

        Human peripheral blood monocytes were isolated
from fresh whole blood using density gradient centrifugation
with Hypaque-Ficoll.   Adherent cells (>85% monocytes) were
cultured in RPMI 1640 medium containing 1 mg/ml bovine serum
5   albumin (BSA) and gentamicin (50 $\mu g/ml$) at a density of 10^6
cells/ml for 18 hr at 37°C in an atmosphere of 95% air and
5% CO_2 in the absence or the presence of 1 $\mu g/ml$ LPS, immune
complex (0.5 mg/ml) or fmet-leu-phe ($10^{-6}M$). Conditioned
media were removed and the attached cells were scraped in
10   phosphate buffered saline and centrifuged at 2000 x g for
10 min.   Peptides were extracted from both the media and
cell pellets by acid ethanol extraction and ether
precipitation as described by Roberts et al. (1980).
Briefly 1 vol of conditioned medium or cell pellet from
15   activated or nonactivated cells was extracted with 2 vol of
acid/ethanol (1 vol/51 vol) overnight at 4°C. After
centrifugation at 600 x g for 30 min. the supernatants were
precipitated with 4 vol of anhydrous ethyl ether overnight
at 4°C.   The precipitates were reextracted with 1 N acetic
20   acid, lyophilized and tested in smooth muscle cell
chemotaxis assay or in Western blots.   In some cases
conditioned medium was tested either directly for
chemotactic activity or lyophilized and resuspended in
sample buffer (Tris, SDS, glycerol) and tested in Western
25   blots.

Characterization of LDGF

        Partial purification of LDGF from activated
monocytes conditioned media was performed by affinity
chromatography on heparin Sepharose (Pharmacia, Piscataway,
30   New Jersey) eluted with a gradient of 0.1-1 N ammonium
acetate, pH7 and size exclusion chromatography (HPLC, TSK-
2000, developed in 1 N acetic acid or 0.5 N ammonium
acetate).

Partially purified material was further
characterized using cyanogen bromide and formic acid
digestion as described by Heldin et al. (1986). Aliquots
were incubated for 48 hr at 37°C in 70% formic acid alone or
70% formic acid containing cyanogen bromide (g CNBr/g total
protein). Samples were then lyophilized, redissolved in
5 mM HCl and tested for chemotaxis and in Western blots.

Chemotactic activity was measured in the Boyden
chamber chemotaxis assay with bovine aortic smooth muscle
cells as described previously (Grotendorst et al., 1981,
1982). Samples were tested in duplicates and each result
represents mean ± S.D. for 3 experiments. Chemotactic
activity is expressed as the milliabsorbance units of stain
extracted from the responding cells as described previously
in detail (Grotendorst, 1987). Pure human PDGF at a
concentration of 6 ng/ml elicits a chemotactic response that
gives a value of 350 mAU at A_{600}nm. Electrophoresis of each
sample was performed on 12 or 15% polyacrylamide gels except
for CNBr and formic acid treated material, which was
analyzed on 18% gels containing sodium dodecylsulfate (SDS)
as described by Laemmli (1970). The proteins were
transferred to nitrocellulose by electroblotting as
described by Kyhse-Andersen (1984). After transfer the
blots were incubated in Tris-buffered saline (TBS) (100 mM
NaCl, 50 mM Tris, pH 7.4) containing 2.5 mg/ml nonfat
powdered carnation milk (TBS-milk) for 4 hr in order to
block nonspecific binding of protein to the filter. The
filters we then incubated overnight in the presence of
15 µg/ml anti-human PDGF IgG diluted in TBS-milk. The
filters were then washed 5 x in TBS-milk (5 min each) and
incubated with alkaline phosphatase conjugated affinity
purified rabbit anti-goat IgG (1:1000 dilution) in TBS-milk
for 90 min. After washing with TBS-milk (5x) the antigens
were detected using an alkaline phosphatase substrate kit

(KPL, Gaithersburg, Maryland). With this method we can
detect as little as 0.3 ng of PDGF in a Western blot.
Non-activated Monocytes Contain PDGF-like
Biological and Immunological Activity

5   Conditioned media from activated peripheral blood
monocytes elicited smooth muscle cell chemotaxis in a
concentration dependent manner whereas conditioned media
collected from nonactivated cells did not contain any
detectable level of chemotactic activity. In contrast, cell
10   extracts from both non-activated and activated cells
contained approximately equivalent amounts of biological
activity suggesting that this material is present within
non-activated cells. The concentration of the material
secreted was as reported (Martinet et al., 1986) time
15   dependent, reaching a maximal concentration after 18 hr and
the response was the same for all the agents used for
activation (i.e., LPS, immune complexes). Also as
previously reported (Shimokado et al., 1985; Martinet et
al., 1986) preincubation of the conditioned media with 30 μg
20   of anti-human PDGF goat IgG completely abolished the
biological activity, whereas non-immune goat IgG had no
effect on the biological activity of the material.
           In order to further characterize the material
produced by the peripheral blood monocytes, cell extracts
25   and conditioned media from both non-activated and activated
cells were examined by Western blot transfer analysis using
anti-human PDGF IgG. Extracts from non-activated monocytes
revealed at least five major immunoreactive peptides with
distinct molecular weights of 56, 45, 31, 25 and 16 kd. In
30   contrast, the activated monocytes contained only one major
immunoreactive peptide of 16 kD identical to the molecular
weight of the secreted product from activated cells.
Conditioned media from non-activated cells did not contain
any detectable immunoreactive material. Preimmune IgG did

not react with any peptides in either of the cell extracts
or the conditioned media.

Mitogenic assay for LDGF

NIH/3T3 cells (ATCC CRL1658) were plated in 24
well plates in Dulbecco's Modified Eagle's Medium (DMEM)/10%
fetal bovine serum (FBS) at a density of $1x10^4/cm^2$, allowed
to grow to confluence and used for assay 3-4 days later.
Growth factors were added directly and after 18 hours ^{3}H-
thymidine (2uCi/ml) (Amersham, Arlington Heights, Illinois)
was added. The cells were incubated an additional 2 hours
and washed at 4°C three times with phosphate buffered saline
(PBS), five times with 5% trichloracetic acid (TCA). TCA
insoluble materials were solubilized in 0.1 N NaOH/0.1% SDS,
and the amount of incorporated 3H-Thymidine was determined
with a Beckman liquid scintillation counter.

LDGF was purified from activated monocytes in
usual manner. PDGF was isolated from platelets as described
by Grotendorst, Cell 36:279-285, 1984.

PDGF	20 ng/ml	71,000 cpm	
PDGF	10 ng/ml	51,000 cpm	
LDGF	100 ul of sample	59,000 cpm	≈14 ng/ml
LDGF	50 ul of sample	45,000 cpm	≈ 7 ng/ml
LDGF	25 ul of sample	28,000 cpm	≈ 3 ng/ml
negative control		2,500 cpm	

LDGF is a Monomer Lacking Interchain Disulfide Bridges

Previous studies by several laboratories have
suggested that only dimeric forms of PDGF are biologically
active (Antoniades et al., 1979; Heldin et al., 1981;
Grotendorst et al., 1981). However, the molecular weight of
the major immunoreactive peptide secreted by the macrophages
as mentioned above was 16 kd as compared to 31 kd for the
platelet released PDGF raising the question of the

22

relationship of the monocyte secreted material to PDGF.
Western blot analysis of the material secreted by the
activated macrophages revealed a doublet of 16 kd on SDS
gels. Reduction of these molecules with dithiothreitol did

5   not alter the electrophoretic mobility of the peptides,
indicating the absence of interchain disulfide bridges. In
marked contrast, electrophoresis on the same gel of
authentic human PDGF isolated from platelets exhibits a
doublet at 31 kd under nonreducing conditions which shifts

10 after reduction to 18 kd as has been reported previously
(Johnsson et al., 1982; Antoniades et al., 1979; Heldin et
al., 1981). The molecular weight and relative abundance of
the immunoreactive peptides secreted by the monocytes was
independent of the agent used to stimulate activation of the

15 cells (LPS, immune complexes, or FMLP; data not shown).
These data indicate that under denaturing conditions the
major secreted form of LDGF behaves as a monomeric protein
of 16 kD on SDS gel electrophoresis.

          The molecular weight of this material was examined
20 under non-denaturing conditions. Using gel filtration
chromatography on HPLC we compared the elution positions of
human PDGF with the monocyte PDGF-like factor (LDGF) on a
TSK-2000 column developed in 1 N acetic acid or 0.5 N
ammonium acetate pH 7. Under acidic conditions the elution

25 times of PDGF and the LDGF were nearly identical, indicating
that the LDGF behaves as a 30-kd dimer under these
conditions. However, the chemotactic activity eluted in 0.5
N ammonium acetate as a 18 kD peptide. Western blot
analysis of the biologically active fractions demonstrated

30 that the 16-kd immunoreactive peptide co-eluted with the
biological activity from this column.
Evidence for the Presence of Additional Methionine
Residues in LDGF Compared to PDGF A or B Chain Molecules

23

Human PDGF A and B chain molecules have
characteristic primary structures which can be exploited by
protein chemical techniques to compare the relationship of
the LDGF to these peptides (Heldin et al., 1986). For
5   example, the mature form of the PDGF A chain molecule does
not contain any methionine residues (Betsholtz et al.,
1986), whereas the mature form of the B chain molecule
contains a single methionine at position 11 (Doolittle et
al., 1983; Waterfield et al., 1983). In addition the
10   processed A chain contains aspartic acid-proline linkages
that are susceptible to cleavage by formic acid digestion.
The mature form of the B chain molecule lacks any of these
linkages (Doolittle et al., 1983; Waterfield et al., 1983).
Thus, B chain molecules are resistant to cleavage with
15   formic acid but are cleaved at position 11 when CNBr is
added, resulting in a peptide of reduced molecular weight
(Heldin et al., 1986). In contrast, A chain molecules are
sensitive to formic acid digestion and CNBr has no
additional effect on the fragmentation of A chain peptides
20   (Heldin et al., 1986). LDGF was then treated with either
formic acid alone or in the presence of CNBr and both the
biological and electrophoretic properties of the treated
molecules were analyzed. Formic acid treatment of LDGF
under conditions which result in cleavage of the PDGF A
25   chain molecule had no effect on either the biological
activity as determined in the smooth muscle cell chemotaxis
assay or in the electrophoretic mobility of the major
immunoreactive peptide in Western blots. However, CNBr
digestion of this material resulted in a complete loss of
30   biological activity as well as completed destruction of the
immunoreactive material on Western blots. In contrast, the
partially purified PDGF B chain molecule isolated from
SSV/NRk cells was easily detected before and after CNBr
digestion in the Western blot assay using anti-PDGF IgG.

24

LDGF is Immunologically Distinct to the
Amino Terminals of PDGF A or B Chain

In order to further determine the relation of LDGF
to PDGF A or B chain antibody was raised against synthetic

5   peptides containing the N-terminal sequences of the secreted
PDGF A (amino acids 92-119) or B (amino acids 79-107) chain.
When LDGF was analyzed and compared to reduced PDGF A and B
homodimers on Western blots, antibody against the amino
terminal peptide of the A chain recognized the reduced PDGF

10   A homodimer but did not react with reduced LDGF or reduced
PDGF B homodimer.   Antibody against the amino terminal
peptide of the B chain immunoreacted with the reduced PDGF B
homodimer and did not crossreact with reduced PDGF A
homodimer or LDGF (Fig. 5).

15   Data presented here indicate that activated human
peripheral monocytes predominantly secrete only one size
class of biologically active peptides of approximate
molecular size of 16 kD.   These peptides also exhibit smooth
muscle cell chemotactic activity, which is completely

20   neutralized by anti-human PDGF antibody.   They appear to be
processed upon activation entirely within the cells, as
extracts of the activated cells contain only peptides with
electrophoretic mobility identical to that of the secreted
peptides and freshly isolated or non-activated cells contain

25   immunoreactive peptides of larger molecular sizes (54, 45,
31, 25, and 16 kd).   Higher molecular weight PDGF related
peptides have been described in various cell lines including
the simian sarcoma virus transformed marmoset line and are
believed to represent precursor forms of the mature molecule

30   that contain additional N and C-terminal amino acids
(Robbins et al., 1983).   LDGF can exist as a dimer
under non-denaturing conditions but lacks any interchain
disulfide bridges.   This is supported by our observations

25

that LDGF and PDGF coelute on HPLC gel filtration
chromatography in acidic condition but exhibit different
electrophoretic mobilities on SDS gels. In contrast to
PDGF, the mobility of LDGF in the SDS gels is unaffected by
the addition of reducing agent. Recent studies by Giese et
al. (1987) have shown that modification by site directed
mutagenesis of any of the eight conserved cysteine residues
in the PDGF-related domain of the v-sis oncogene results in
the synthesis of forms of the v-sis gene product (PDGF B
chain) that lacks interchain disulfide bridges. However,
modification of the cysteine residues at positions 154, 163,
164 and 210 did not alter the transforming activity of the
v-sis gene, indicating that certain forms of the v-sis gene
product lacking interchain disulfide bridges retain
biological activity. Unlike the PDGF A or B chain peptides,
the immunoreactivity of the LDGF was completely destroyed by
treatment with cyanogen bromide suggesting the presence of
additional methionine residues. Also antibody specifically
recognizing the amino terminal sequences of the PDGF A and B
chain failed to react with LDGF indicating that such amino
acid sequences are absent in LDGF, or are significantly
altered or not accessible to the antibody, further
supporting structural differences between LDGF and PDGF.

          In summary, these data suggest that the structure
of LDGF is distinct from any of the forms of PDGF which have
been characterized to date. This material appears to
exhibit all of the PDGF-like biological activities including
action as a connective tissue cell chemoattractant and
mitogen as well as competition with [^{125}I] PDGF for binding
to its cell surface receptor. However, its molecular
organization appears to be different from that of the known
forms of the PDGF in that it is not an interchain disulfide
cross-linked dimer and exhibits different sensitivities to
cleavage by formic acid or by CNBr than has been shown for

either the processed PDGF A or B chain peptides (Heldin et al., 1986).

The purified material appears to have biological activity at concentrations of 10-40 ng/ml which is at the $10^{-9}M$ range of concentration. This is comparable to the molar concentration of PDGF which are required for similar biological activity.

Example 2: Immunodetection of PDGF-Related Peptides in Human Wound Fluid

METHODS AND MATERIALS

Cells. NIH/3T3 cells were obtained in early passage from S. Aaronson (National Institutes of Health). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and gentamicin (50 μg/ml) and maintained at 37°C in 90% atmosphere/10% CO_2. All mitogenic assays and chemotaxis assays were done by using density-inhibited cultured cells just after they had become confluent.

Human Wound Fluid. Human wound fluids were collected daily from six female patients who had radical mastectomies for tumor (diameter, <1.5 cm) removal. Fluid was collected through the suction tubing placed during surgery for drainage as a customary procedure. None of these patients exhibited any abnormal hematological or cell-mediated immunological variables. Usual postoperative management consisted of drop infusions of antibiotics and vitamins and hemostatic agents such as adrenochrome and tranexamic acids. No anticancer agents were administered during the collection of wound fluids. Wound fluid was separated from tissue debris by centrifugation (10 min at 5000 x g) and stored at -20°C.

Growth Factors. Genetically manufactured PDGF AA homodimer, BB homodimer, AB heterodimer, and human epidermal

27

growth factor were supplied by Creative BioMolecules
(Hopkinton, MA). The concentration and purity were
determined by amino acid sequence analysis. Human
transforming growth factor type ß (TGF-ß) was purchased from
5   Research & Diagnostic Systems (Minneapolis, MN). Acidic
fibroblast growth factor was purchased from Sigma. In some
experiments PDGF purified from platelets, as described
(Grotendorst, 1984), was used as a standard and immunogen.
Macrophage-derived growth factor was purified by HPLC as
10   reported (Pencev and Grotendorst, 1988) from culture
supernatant of activated human macrophages.

          Antibodies. Anti-human PDGF and anti-synthetic
peptide of PDGF A- and B-chain components were used. Goat
anti-human PDGF antibody was raised against purified PDGF
15   from platelets (Grotendorst, 1984). This goat anti-PDGF
antibody can specifically neutralize the biological activity
of PDGF (Grotendorst, et al., 1988).

          Anti-A-chain- or anti-B-chain-specific antibodies
were prepared by using synthetic peptides containing the
20   amino-terminal sequences of the PDGF A chain (amino acids
92-119) of the precursor molecule) and the PDGF B chain
(amino acids 79-107) as immunogens. Immune sera against the
PDGF A (92-119) and B (79-107) peptide proved to be sequence
specific as determined by immunodot blot and did not
25   crossreact with peptides containing the remaining amino acid
sequences of the PDGF A- or PDGF B-chain molecule.
Additionally, the antisera are chain specific and react only
with the PDGF A- or B-chain peptide, respectively.

          Purification of PDGF-Related Peptide in Human
30   Wound Fluid. Due to the high protein concentration the
wound-fluid samples or acid-extracted samples cannot be
analyzed by Western blot (immunologic) techniques; therefore
PDGF-related peptides in human-wound fluid were purified by
anti-PDGF immunoaffinity techniques. Goat polyclonal anti-

PDGF IgG was conjugated to Affi-Gel-10 (Bio-Rad) as
indicated by the manufacturer's protocol. PDGF-related
peptides were isolated by incubation of 1 ml of wound fluid,
freshly thawed from frozen stock, for 24 hr at 4°C with

5   200 µl of Affi-Gel-10 conjugated with anti-PDGF IgG. After
incubation with the wound fluid, the matrix was washed five
times with 1 ml of 0.1 M Hepes, pH 7.4. The specifically
bound peptides were released by treatment with 1 M acetic
acid for 24 hr. The supernatant was then harvested by

10   centrifugation of the matrix and dialyzed against 1.0 M
acetic acid and stored at -20°C before assay. Identical
amounts of wound fluid and affinity matrix were used to
isolate the PDGF-related peptides from each sample.
Preliminary studies showed that this amount of affinity

15   matrix is in >10-fold excess for recovering all PDGF-related
peptides in the wound-fluid samples. In some experiments
human wound fluid was processed by acid ethanol
precipitation as described by DeLarco and Todaro (1978).
       Western Blot Analysis. Human wound-fluid

20   materials were analyzed by Western blot analysis. Samples
were electrophoresed under nonreducing conditions, except in
those cases in which anti-peptide antibodies were used in a
15% polyacrylamide gel with SDS, and samples were then
electroblotted to nitrocellulose as described (Leibovich and

25   Ross, 1976; Takehara, et al., 1987). Immunoreactive PDGF
peptides were detected with anti-human PDGF IgG and alkaline
phosphate-conjugated rabbit anti-goat IgG.
       Mitogenic and Chemotactic Assays. The mitogenic
activity of purified wound-fluid samples was determined as

30   follows. NIH 3T3 cells (Grotendorst, 1984) were cultured in
48-well plates (Costar) in DMEM with 10% fetal calf serum
until confluent, and the medium was changed to DMEM with
2.5% fetal calf serum 12 hr before use. Samples in DMEM
were added to each well, and DNA synthesis was measured 16

hr later by [3H]thymidine incorporation into trichloroacetic
acid-precipitable material.

The chemotactic assays were performed in modified
Boyden chambers as described (Grotendorst, 1987) by using
5   collagen-coated polycarbonate filters (Nuclepore; 8-µm-
diameter pores). Assays were run for 4 hr in serum-free
DMEM containing bovine serum albumin at 2.0 mg/ml. The
chemotactic response was quantiated by measuring the
absorbance at 600 nm of stain extracted from cells that
10   migrated through the filter.

30

RESULTS
PDGF-Related Peptide in Human Wound Fluid.

Western blot analysis of PDGF-related peptides in human
wound fluid revealed two different molecular mass species
5   (16-17 kDa and 34-36 kDa). The Western blot clearly shows
that the 16- and 17-kDa peptides are most concentrated in
the fluid on the first day after surgery and then decrease
by day-7 postsurgery. In contrast, the high-molecular-mass
peptides (34-36 kDa) are present in trace quantities at the
10   initial stage of the wound healing process, increase by days
4 and 5, and then decrease by day 7. These findings were
essentially the same for each of the six patients whose
wound fluid was examined. The 16- to 17-kDa product appears
to be derived from macrophages, as it comigrates with a
15   macrophage-derived growth factor (Pencev and Grotendorst,
1988) standard purified from activated macrophages. The 34-
to 36-kDa product does not comigrate with PDGF, and we are
uncertain of the origin of this peptide.

          Specificity of the polyclonal anti-human PDGF is
20   demonstrated by the blocking study of antibody with
recombinant human AB PDGF. Preincubation of the first
antibody with 50 ng of AB heterodimer completely blocks the
binding of that antibody to the PDGF-related peptides in
wound fluid. This phenomenon was also seen by using AA or
25   BB homodimers as a blocker. Nonimmune IgG does not detect
any peptides in this assay system. Furthermore, this
polyclonal anti-human PDGF is specific for PDGF and does not
react with TGF-ß (100 ng), human epidermal growth factor
(100 ng), or acidic fibroblastic growth factor (100 ng). The
30   wound fluid was red in color during the first 3 days of
collection, indicating that bleeding and coagulation
occurred at the wounding site. Surprisingly, in spite of
the platelet activation occurring at the site, we could not
detect any authentic 30-kDa PDGF in any sample from the six

31

sets of wound-fluid samples examined.

Efficacy of Anti-PDGF Immunoaffinity Purification.
Several explanations for the inability to recover authentic
30-kDa PDGF are (i) 30-kDa PDGF has been degraded by
enzymatic digestion, (ii) the immunoaffinity-purification
procedure is not effective enough to pick up the antigen
from the protein-rich wound fluid, or (iii) 30-kDa PDGF is
bound to carrier proteins and is therefore inaccessible to
the antibody. To address these questions, a known amount of
PDGF (20 ng/ml) was added into the wound fluid. The sample
was incubated for 24 hr at room temperature, and the PDGF-
related peptide was purified by the aforementioned
immunoaffinity procedure. Recovery of the exogenously added
PDGF was >90%, and the amount of immunoreactive peptides
present in the wound fluid did not differ with or without
addition of authentic PDGF. These results strongly argue
that any PDGF present in the wound fluid would be
recoverable with this isolation procedure and that the
amount of matrix used is in excess, as doubling the amount
of PDGF antigen in the fluid sample had no effect on
recovery of PDGF or PDGF-related peptides as judged by the
Western blot assay.

Absence of PDGF A- and B-Chain Peptides in Human
Wound Fluid. To examine whether the PDGF-related peptide in
the wound fluid was composed of PDGF A or B chains larger
amounts of purified PDGF-related peptides from day-1 wound
fluid were analyzed using antisera specific for the PDGF A
or B chain. These sera were produced against 30-mer
synthetic peptides that correspond to the N-terminal
sequence of either the mature A- or B- chain molecule.
These antisera are chain specific and can detect as little
as 1 to 2 ng of either the intact A- or B-chain peptide.
Neither antisera detected any A- or B-chain peptides in a
sample of day-1 wound fluid that contained the equivalent of

32

20 ng of PDGF biological activity. These results suggest
that PDGF A- or B-chain peptides must be present in
concentrations of <2 ng/ml in the wound fluid and that the
principal PDGF-related biological activity cannot be
5   attributed to A- or B-chain molecules in the wound fluid.

Biological Activity of PDGF-Related Peptide in
Human Wound Fluid. The amount of PDGF-related peptides was
determined by using both densitometric scanning of the
Western peptide blots and biological assays. The kinetics
10   of appearance and disappearance of the 16- to 17-kDa and 34-
to 36-kDa product were independent of each other. The
level of the 16- to 17-kDa peptide peaked on the first day
after surgery and decreased exponentially to nearly
undetectable levels by the seventh day. In contrast, the
15   34- to 36-kDa product was initially present at low levels
and then increased, reaching peak levels on the fifth and
sixth days postsurgery.

The chemotactic and mitogenic activity of the
total wound fluid and immunoabsorbed fraction was
20   investigated. The mitogenic activity of the immunoaffinity-
purified fraction was highest on day 1 and decreased to
undetectable levels after the fourth day postsurgery. These
kinetics are identical to that of the 16- to 17-kDa peptide
as detected in the Western blot analysis. The mitogenic
25   activity of the total wound fluid differed from that of the
immunoaffinity-purified fraction; this activity peaked on
the fifth day, at which time the total mitogenic activity
was greater than that of immunoaffinity-purified material.
Thus, multiple mitogenic activities are present in the wound
30   fluid, only some of which immunologically relate to PDGF.

The chemotactic activity of the total wound fluid
and the immunoaffinity-purified fraction was also
investigated. As for mitogenic activity, the amount of
chemotactic activity in the immunoaffinity-purified fraction

correlated with the day-to-day change of the 16- to 17-kDa
peptide as measured by Western blot analysis. This activity
peaked on the first day and then decreased.

Chemotactic activity of the purified PDGF-related
peptides from wound fluid was treated with recombinant PDGF
in a competitive assay (Table 1). PDGF-related peptides
from day-1 wound fluid elicited a strong chemotactic
response by NIH 3T3 cells without recombinant PDGF BB
homodimer in the upper chamber. However, when PDGF BB
homodimer at 25 ng/ml was added to the upper chamber,
decreasing the concentration gradient of chemoattractant, no
cells migrated toward the lower chamber in response to the
immunopurified factor. These data indicated that the
immunopurified chemotactic factor is probably acting through
the PDGF receptor.

The activity present in acid ethanol extracts of
wound fluid exhibited kinetics different from those of the
immunoaffinity-purified PDGF-related peptides, as the
activity was at its peak on the fourth day of postsurgery.
Thus, as with the mitogenic activity, multiple chemotactic
activities appear to be present, some that are PDGF like and
others that are not.

The PDGF-related chemotactic and mitogenic
activities appear to be primarily mediated by a factor that
resembles a macrophage-derived PDGF-related peptide and not
authentic platelet PDGF (Pencev and Grotendorst, 1988).
Whether this factor is exclusively produced by macrophages
is not yet clear. Recent work in my laboratory indicates
neutrophils can produce a similar factor, although at
present the relationship of this factor with macrophage-
derived growth factor is unknown. Nonetheless, macrophages
have long been felt to play a central role in controlling
the wound repair response (Leibovitch and Ross, 1975;
Diegelmann, et al., 1980), and it is not unexpected to find

abundant levels of an apparent macrophage-related product in
the wound fluid.   The absence of any authentic PDGF peptides
in the wound fluid was surprising; whether these peptides
are present in amounts below assay sensitivities or only
5   present in trace quantities is unclear.   If authentic PDGF
peptides are present in low levels in the wound fluid, they
must account for <10% of the total PDGF-related biological
activity.   That the authentic PDGF is masked in some manner
or is degraded during the aforementioned isolation
10   procedures is unlikely, as added PDGF is recovered with >90%
efficiency from the wound-fluid samples.   Possibly the PDGF
A- and B-chain peptides are predominantly associated with
the extracellular matrix or fibrin clot and are therefore
not free in solution.   Alternatively, the authentic PDGF
15   could be rapidly consumed at the site of tissue repair by
tissue elements.

A significant portion of the chemotactic and
mitogenic activities in the wound fluid collected during the
later stages of the repair response remained after depletion
20   of the PDGF-related peptides by immunoabsorption.   The
mitogenic and chemotactic activities in untreated wound
fluid or acid ethanol extracts, which resemble each other,
exhibit different time courses of appearance than those of
the immunoaffinity-purified materials.   These activities
25   reach peak levels on later days, when the level of 16- to
17-kDa PDGF-related peptide has decreased, indicating that
these factors are probably produced by different cell types
than those producing the PDGF-related peptides.   Thus,
mitogenic and chemotactic factors distinct from PDGF are
30   also present in wound fluid and may play essential roles in
regulating later stages of the repair process.

These results show the presence of PDGF-related
peptides at sites of normal wound healing in humans.
Similar peptides have been found in both ascites fluid and

liver biopsy tissue obtained from individuals with chronic
alcoholic cirrhosis. Because this disorder is characterized
by chronic inflammation of the liver and a large
accumulation of mononuclear cells at this site, macrophage-
5   derived growth factor is a probable component of the
initiation of fibrotic diseases as well. Thus, it appears
that cell types, such as macrophages, may figure importantly
in the production of PDGF-related growth factors during the
normal repair and regeneration of tissue after traumatic
10   injury and that similar factors may stimulate connective-
tissue formation during fibrotic disorders such as
atherosclerosis, arthritis, pulmonary fibrosis, and liver
cirrhosis.

Example 3:   Isolation and Characterization
15   of LDGF-Related cDNA

        Total RNA from activated (LPS treated [1/ug/ml] 24
hours) human peripheral blood monocytes was prepared by the
method of Chugauin et al.; (Biochemistry; 18:5294-5299;
1979). The poly A+ containing mRNA was isolated by oligo dt
20   cellulose chromatography. A cDNA copy of all the mRNAs
present was constructed using a cDNA library construction
kit purchased from Pharmacia. The cDNAs were then blunt
ended and had EcoR1 adaptors added to the ends. The cDNAs
were isolated by low melt agarose electrophoresis and all of
25   the cDNA's larger than 800 base pairs in length were
collected by low melt agarose electrophoresis and packaged
into a lambda gt 11 expression vector. This genetic vector
packages the cDNA insert in a position so that it is under
the control of a beta galactosidase promoter and when the
30   appropriate stimulus is added to the bacterial culture media
this promoter activates the synthesis of a fusion protein
which contains a N-terminal of beta-galactosidase and the C-
terminal of the protein sequence encoded by the cDNA
packaged into that phage genome. Only a single cDNA

sequence is packaged per phage genome.

The expression library was then screened with the anti-PDGF antibody we had prepared in goats and used to identify LDGF in the conditioned media. A total of 800,000 recombinant phage were screened and a single phage produced a fusion protein which reacted with the antibody. This phage was plaque purified and the insert cut out using Eco R1 restriction endonuclease. It was evident from our restriction fragments that the insert contained an internal EcoR1 site at approximately 300 bases in from one of the EcoR1 linkers.

The intact insert was then subcloned in M13 using partial digestion with EcoR1 and screening a large number of recombinants for the intact insert. Several plaques were picked for single strand DNA sequencing using the Dideoxy method. DNA sequence analysis revealed an insert of 675 base pairs which contained a single open reading frame of 384 bases (Figure 1) and the predicted internal EcoR1 site. A search of the Genbank library with this sequence revealed that this was a unique sequence and showed a 40% homology to the protein encoded by the gro gene (see Table 2). This indicated that the cDNA was related to the CTAP-III gene family. The open reading frame was translated using the PC-GENE program and compared with protein sequences in the data base. The protein sequence showed regions of identity with three known proteins, Platelet-basic protein (PBP), CTAP III or LA-PF4, and beta-thromboglobulin (βTG). However, the predicted LDGF sequence contained an additional 34 amino acids at the N-terminal of the PBP-related sequence. CTAP-III and βTG are believed to be proteolytic breakdown products of PBP. In fact beta-thromboglobulin is used as a marker for outdating platelets. In our tests neither PBP, LA-PF4, or βTG displayed any biological or immunological activity. Thus, it appears that the additional amino acids

present in the N-terminal of the LDGF protein are essential
for its biological activity. Using microcomputer programs
(GENEPRO, Riverside Scientific), it has been shown that
there is a 25% homology between LDGF and regions of the PDGF
5   B chain molecule if one includes substitutions of certain
amino acids which are neutral to the molecular structure.
(see Table 3). Importantly, 3 of the 5 cysteine residues in
the LDGF sequences are aligned with cysteines in the PDGF B
chain protein. Furthermore, comparison of hydropathy slots
10 which reflect the hydrophobic and hydrophilic regions of
LDGF and PDGF-B chain indicate that the intact LDGF molecule
has a very similar profile as seen in PDGF. These
similarities may explain the basis for the antibody and
receptor binding cross reactivity, and suggests there are
15 loops of the molecule which may share regions of structural
identity.

   LDGF is produced by both activated macrophages and
neutrophils. Furthermore, when fluid collected from healing
surgical wounds in humans was analyzed it was found that
20 LDGF was the principal PDGF-like factor in the wound fluid
and authentic PDGF was absent (Example 2). LDGF is present
in all types of normal healing wounds including skin wounds
and orthopedic surgical wounds (bone fusion).

   Importantly, wound fluid collected from non-
25 healing skin ulcers which occur in diabetic patients,
steroid treated rheumatoid patients and individuals who have
peripheral circulatory problems has been analyzed and, in
all cases examined to date, neither PDGF-like biological
activity nor LDGF immunoreactivity has been detected in
30 these samples using equivalent amounts as those tested from
the normal healing individuals. These results implicate a
deficiency of LDGF at sites of injury in individuals with
healing impairments. This material was also present in
liver tissue of patients with chronic alcoholic cirrhosis

38

but not in normal liver tissue or tissue from diseased livers which do not develop fibrotic complications such as hepatoma.

REFERENCES

Antoniades, H.N., Scher, C.D., and Stiles, C.D. (1979). Purification of human platelet derived growth factor. Proc. Natl. Acad. Sci. (USA), 76 1809-1813.

Baird, A., Mornede, P., and Bohlen, P. (1985). Immunoreactive fibroblast growth factor in cells of peritoneal exudate suggests its identity with macrophage derived growth factor. Biochem. Biophys. Res. Commun., 126, 358-364.

Barret, T.B., Gajdusek, C.M., Schwartz, S.M., McDougall, J.K. & Benditt, E.P. (1984) Proc. Natl. Acad. Sci. (USA), 81, 6772-6774.

Betsholtz, C., Johnsson, A., Heldin, C.H., Westermark, B., Lind, P., Urdea, M.S., Eddy, R., Shows, T.B., Philpott, K., Mellor, A.L., Knott, T.J., and Scott, J. (1986). cDNA sequence and chromosomal localization of human platelet derived growth factor A-chain and its expression in tumor cell lines. Nature, 320, 695-699.

Bittermann, P.B., Rennard, S.I., Hunninghake, G.W., and Crystal, R.G. (1982). Human alveolar macrophage growth factor for fibroblasts: regulation and partial characterization. J. Clin. Invest., 70, 806-822.

Collins, T., Bonthron, D.T., and Orkin, S.H. (1987). Alternative RNA splicing affects function of encoded platelet-derived growth factor A-chain. Nature, 328, 621-624.

DeLarco, J.E. & Todaro, G.J. (1978) Proc. Natl. Acad. Sci. (USA), 75, 4001-4005.

DeLustro, F., Sherer, S.K., and LeRoy, E.C. (1980). Human monocyte stimulation of fibroblast growth by

39

a soluble mediator(s). J. Reticuloendoethel. Soc., 28: 519-532.

Diegelmann, R.E., Cohen, I.K. & Kaplan, A.M. (1980) Plastic Reconstr. Surg., 68, 107-113.

Doolittle, R.F., Hunkapiller, M.W., Hood, L.E., Devare, S.G., Robbins, K.C., Aaronson, S.A., and Antoniades, H.N. (1983). Simian sarcoma virus onc gene, v-sis, is derived from the gene (or genes) encoding a platelet derived growth factor. Science, 221, 275-277.

Douglass, J., Cirelli, O., and Herbert, D. (1984). Protein gene expression: generation of diversity of neuroendocrine peptides, Annu. Rev. Biochem., 53, 665-671.

Estes, J.E., Pledger, W.J., and Gillespie, G.Y. (1984). Macrophage-derived growth factor and interleukin 1 are distinct entities. J. Leuk. Biol., 35, 115-129.

Giese, N.A., Robbins, K.C., and Aaronson, S.A. (1987). The role of individual cysteines residues in the structure and function of the v-sis gene product. Science, 236, 1315-1318.

Glenn, K., and Ross, R. (1981). Human monocyte-derived growth factor(s) for mesenchymal cells: activiation of secretion by endotoxin and concavalin A. Cell, 25, 603-615.

Grotendorst, G.R., Pencev, D., Martin, G.R. & Sodek, Jr. (1984) in Soft and Hard Tissue Repair, eds. Hunt, T.K., Heppenstall, R.B., Pines E. & Rovee, D. (Praeger, New York), pp. 20-44.

Grotendorst, G.R. (1984). Alteration of the chemotactic response of NIH/3T3 cells to PDGF by growth factors, transformation and tumor promoters. Cell, 36, 279-285.

Grotendorst, G.R. (1987). Spectrophotometric assay for the quantitation of cell migration in the Boyden chamber chemotaxis assay. Methods Enzymol., 147, 144-152.

40

Grotendorst, G.R., Harvey, A.K., Nagarajan, L., Anderson, W.B. & Gatewood, E. (1988) J. Cell Physiol., 134, 437-444.

Grotendorst, G.R., Seppa, H.E., Kleinman, H.K., and Martin, G.R. (1981). Attachment of smooth muscle cells to collagen and their migration toward platelet derived growth factor. Proc. Natl. Acd. Sci. (USA), 78, 3669-3672.

Grotendorst, G.R., Chang, T., Seppa, H.E., Kleinman, H.K., and Martin, G.R. (1982). Platelet derived growth factor is a chemoattractant for vascular smooth muscle cells. J. Cell Physiol., 113, 261-266.

Grotendorst, G.R., Seppa, H.E., Kleinmann, H.K. & Martin, G.R. (1981) Proc. Natl. Acad. Sci. (USA), 78, 3669-3672.

Heldin, C.H., Westermark, B., and Wasteson, A. (1981). Platelet-derived growth factor: isolation by a large scale procedure and analysis of subunit composition. Biochem. J., 193, 907-913.

Heldin, C.H., Johnsson, A., Wennegren, S., Wernstedt, C., Betsholtz, C., and Westermark B. (1986). A human osteosarcoma cell line secretes a growth factor structurally related to a homodimer of PDGF A-chains. Nature, 319, 511-514.

Johnsson, A., Heldin, Ch.H., Westermark, B., and Wasteson, A. (1982). Platelet derived growth factor: identification of constituent polypeptide chains. Biochem. Biophys. Res. Commun., 104, 66-74.

Kyhse-Andersen, J. (1984). Electroblotting of multiple gels: a single apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. J. Biochem. Biophys. Methods, 10, 203-209.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680-685.

Lawrence W.T., Sporn, M.B., Gorshboth, C., Norton, J.A. & Grotendorst, G.R. (1986) Ann. Surg. 203, 142-147.

Leibovich, S.J. & Ross, R. (1975) Am. J. Pathol., 84, 71-100.

Leibovich, S.J., and Ross, R. (1976). A macrophage dependent factor that stimulates the proliferation of fibroblasts in vitro. Am. J. Pathol., 84, 501-554.

Martinet, Y., Bitterman, P.B., Mornex, J.-F, Grotendorst, G.R., Martin, G.R. & Crystal, R.G. (1986). Activated human monocytes express the c-sis proto-oncogene and release a mediator showing PDGF-like activity. Nature (London), 319, 158-160.

Mornex, J.-F., Martinet, Y. Yamauchi, K., Bitterman, P.B., Grotendorst, G.R., Chytil-Weir, A., Martin, G.R., and Crystal, R.G. (1986). Spontaneous expression of the c-sis gene and release of a platelet derived growth factorlike molecule by human alveolar macrophages. J. Clin. Invest., 78, 61-66.

Pencev, D. & Grotendorst, G.R. (1988) Oncogene Res., 3, 333-342.

Pledger, W.J., Stiles, C.D., Antoniades, H.N. & Scher, C.D. (1977) Proc. Natl. Acad. Sci. (USA), 74, 4481-4485.

Roberts, A.B., Lamb, L.C., Newton, D.L., Sporn, M.B., DeLarco, J.E., and Todaro, G.J. (1980). Transforming growth factors: isolation of polypeptides from virally and chemically transformed cells by acid/ethanol extraction. Proc. Natl. Acad. Sci. (USA), 77 3494-3498.

Robbins, K.C., Antoniades, H.N., Devare, S.G., Hunkapiller, M.W., and Aaronson, S.A. (1983). Structural and immunological similarities between simian sarcoma virus gene products and human platelet derived growth factor. Nature, 305, 605-608.

42

Ross, R. & Benditt, E.P. (1961) J. Biophys.
Biochem. Cytol., 11, 665-677.

Schmidt, J.A., Mizel, S.B., Cohen, D., and Green
I. (1982). Interleukin 1, a potential regulator of
fibroblast proliferation. J. Immunol., 128, 2177-2182.

Seppa, H.E., Grotendorst, G.R., Seppa, S.,
Schiffmann, E. & Martin, G.R. (1982) J. Cell Biol., 92, 584-
588.

Shimokado, K., Raines, E.W., Madtes, D.K.,
Barrett, T.B., Benditt, E.P., and Ross, R. (1985). A
significant part of macrophage-derived growth factor
consists of at least two forms of PDGF. Cell, 43, 277-286.

Takehara, K., Grotendorst, G.R., Silver, R. &
Leroy, E.C. (1987) Arteriosclerosis, 7, 152-158.

Tong, B.D., Auer, D.E., Jaye, M. Kaplow, J.M.,
Ricca, G., McConathy, E., Drohan, W., and Deuel, T.F.
(1987). cDNA clones reveal differences between human glial
and endothelial cell platelet derived growth factor A-
chains. Nature, 328, 619-621.

Tsukamoto, Y., Helsel, W.E., and Wahl, S.M.
(1982). Macrophage production of fibronectin, a
chemoattractant for fibroblasts. J. Immunol., 127, 673-678.

Vogel, A.E., Raines, E., Kariya, B., Rivest, M.-J.
& Ross, R. (1978) Proc. Natl. Acad. Sci. (USA), 75, 2810-
2814.

Waterfield, M.D., Scrace, G.T., Whittle, N.,
Stroobant, P., Johnsson, A., Wasteson, A., Westermark, B.,
Heldin, C.H., Huang, J.S., and Deuel, T.F. (1983).
Platelet-derived growth factor is structurally related to
the putative transforming protein p28sis of simian sacroma
virus. Nature, 304, 35-39.

**TABLE 1: BLOCKING OF CHEMOTACTIC ACTIVITY OF WOUND FLUID
WITH RECOMBINANT PDGF BB HOMODIMER**

Factor		Stimulation of cell migration,
Lower chamber	Upper Chamber	-fold
PDGF BB (10 ng/ml)	None	3.7
Affinity-purified day-1 wound fluid	None	3.8
PDGF BB (10 ng/ml)	PDGF BB (25 ng/ml)	0.9
Affinity-purified day-1 wound fluid	PDGF BB	0.9
PDGF BB (10 ng/ml)	EGF (25 ng/ml)	3.5
PDGF BB (10 ng/ml)	FGF (25 ng/ml)	3.3
PDGF BB (10 ng/ml)	TGF-ß (25 ng/ml)	3.9
None	None	1.0

Chemotaxis was assayed as described. Neutralization of
the positive PDGF or wound-fluid factor concentration gradient
toward the lower chamber by adding PDGF to the upper chamber
blocked the cell migration to the lower chamber. Other growth
factors including epidermal growth factor (EGF), acidic
fibroblast growth factor (FGF), and TGF-ß at concentrations to
25 ng/ml did not block migration of the cells to PDGF.

44

TABLE 2

Homology of Gro Gene Product with LDGF


```
MARAALSAAPSNPRLLRVALLLLLLVAAGRRAAGASVA-------------TE    40
         ::  . ::: :        .          .:            :
MSLRLDTTPSCNSARPLHALQVLLLLSLLLTALASSTKGQTKRNLAKGKEESLDSDLYAE    60

LRCQCLQTLQGIHPKNIQSVNVKSPGPHCAQTEVIATLKNGRKACLNPASPIVKKIIEKM   100
:::  :. :   :::::::::.  :    :  ::  :  ::::::: :::  ::  :   :  .::: :
LRCMCIKTTSGIHPKNIQSLEVIGKGTHCNQVEVIATLKDGRKICLDPDAPRIKKIVQKK   120

LNSDKSN                      107
:   :  :
LAGDESAD                     128
Aligned 119, Matches 48, Mismatches 71, Homology 40%
```

TABLE 3

Homology of PDGF B-Chain Mature Protein with LDGF


```
SLGSLTIAEPAMIAECKTRTEVFEISRRLIDRTNANFLVWPPCVEVQRCSGCCNNRNLQC    60
                            :  :          .           :
                    MSLRLDTTPSCNSARPLHALQVLLLLSL    28

RPTQVQLRPVQVRKIEIVRKKPIFKKATVTLEDHLACKC-ETVAAARPVT          109
:  .        :   .  .:       :  :  :   :   . .   :
LLTALASSTKGQTKRNLAKGKEESLDS--DLYAELRCMCIKTTSGIHPKNIQSLEVIGKG    86

THCNQVEVIATLKDGRKICLDPDAPRIKKIVQKKLAGDESAD
Aligned 78, Matches 12, Mismatches 66, Homology 15%        128
```

CLAIMS

1.   A non-naturally occurring polypeptide analogue of
LDGF having chemotactic and/or mitogenic activity and have a
greater degree of homology with LDGF than with PDGF, PBP,
CTAP-III or β-TG.

2.   A cDNA coding for a polypeptide having chemotactic
and/or mitogenic activity on fibroblast cells and comprising
an amino acid sequence substantially homologous with the
sequence:
MetSerLeuArgLeuAspThrThrProSerCysAsnSerAlaArgProLeuHisAlaLeu
GlnValLeuLeuLeuLeuSerLeuLeuLeuThrAlaLeuAlaSerSerThrLysGlyGln
ThrLysArgAsnLeuAlaLysGlyLysGluGluSerLeuAspSerAspLeuTyrAlaGlu
LeuArgCysMetCysIleLysThrThrSerGlyIleHisProLysAsnIleGlnSerLeu
GluValIleGlyLysGlyThrHisCysAsnGlnValGluValIleAlaThrLeuLysAsp
GlyArgLysIleCysLeuAspProAspAlaProArgIleLysLysIleValGlnLysLys
LeuAlaGlyAspGluSerAlaAsp

3.   A recombinant DNA molecule comprising a structural
gene coding for a polypeptide having chemotactic and/or
mitogenic activity on fibroblast cells and comprising an
amino acid sequence substantially homologous with the
sequence:
MetSerLeuArgLeuAspThrThrProSerCysAsnSerAlaArgProLeuHisAlaLeu
GlnValLeuLeuLeuLeuSerLeuLeuLeuThrAlaLeuAlaSerSerThrLysGlyGln
ThrLysArgAsnLeuAlaLysGlyLysGluGluSerLeuAspSerAspLeuTyrAlaGlu
LeuArgCysMetCysIleLysThrThrSerGlyIleHisProLysAsnIleGlnSerLeu
GluValIleGlyLysGlyThrHisCysAsnGlnValGluValIleAlaThrLeuLysAsp
GlyArgLysIleCysLeuAspProAspAlaProArgIleLysLysIleValGlnLysLys
LeuAlaGlyAspGluSerAlaAsp, and a promoter operably linked to
the structural gene.

46

4.   The molecule of claim 3 wherein the promoter is not natively associated with said gene.

5.   A culture of cells transformed with a recombinant DNA molecule according to claim 3.

6.   A process for producing a polypeptide having chemotactic and/or mitogenic activity which comprises cultivating a culture of transformed cells according to claim 5 under conditions conducive to expression of said polypeptide.

7.   The polypeptide of claim 1, further characterized by having chemotactic and mitogenic activity at a concentration of less than 1 mg/ml.

8.   The polypeptide further characterized by having sensitivity to cyanogen bromide and being unaffected by formic acid.

9.   A wound healing composition comprising the polypeptide of claim 1 and a pharmaceutically acceptable carrier.

10.   The polypeptide of claim 1, differing from native LDGF at least by the modification of a protease cleavage site so as to render the polypeptide less susceptible to proteolytic degradation than native LDGF.

11.   The polypeptide of claim 10 wherein the serines at position 35 and 36 of native LDGF are mutated.

12.   The polypeptide of claim 10 wherein the asparagine at position 44 of native LDGF is mutated.

13. A recombinant DNA molecule comprising an expressible gene coding for the polypeptide analogue of claim 1.

14. A process for producing a polypeptide having chemotactic and/or mitogenic activity which comprises cultivating a culture of cells transformed by a recombinant DNA molecule according to claim 13 under conditions conducive to expression of said polypeptide.

15. The process of claim 6 wherein the polypeptide comprises an amino acid sequence substantially homologous with the LDGF sequence set forth in Figure 1.

FIG. 1.

```
                    G AGG CAA CTC ACC CTC ACT CAG AGG TCT TCT GGT  34

TCT GGA AAC AAC TCT AGC TCA GCC TTC TCC ACC ATG AGC CTC AGA  79
                                            Met Ser Leu Arg

CTT GAT ACC ACC CCT TCC TGT AAC AGT GCG AGA CCA CTT CAT GCC 124
Leu Asp Thr Thr Pro Ser Cys Asn Ser Ala Arg Pro Leu His Ala

TTG CAG GTG CTG CTG CTT CTG TCA TTG CTG CTG ACT GCT CTG GCT 169
Leu Gln Val Leu Leu Leu Leu Ser Leu Leu Leu Thr Ala Leu Ala

TCC TCC ACC AAA GGA CAA ACT AAG AGA AAC TTG GCG AAA GGC AAA 214
Ser Ser Thr Lys Gly Gln Thr Lys Arg Asn Leu Ala Lys Gly Lys
△Platelet Basic Protein          △LAPF-4/CTAPIII  △BTG
GAG GAA AGT CTA GAC AGT GAC TTG TAT GCT GAA CTC CGC TGC ATG 259
Glu Glu Ser Leu Asp Ser Asp Leu Tyr Ala Glu Leu Arg Cys Met

TGT ATA AAG ACA ACC TCT GGA ATT CAT CCC AAA AAC ATC CAA AGT 304
Cys Ile Lys Thr Thr Ser Gly Ile His Pro Lys Asn Ile Gln Ser

TTG GAA GTG ATC GGG AAA GGA ACC CAT TGC AAC CAA GTC GAA GTG 349
Leu Glu Val Ile Gly Lys Gly Thr His Cys Asn Gln Val Glu Val

ATA GCC ACA CTG AAG GAT GGG AGG AAA ATC TGC CTG GAC CCA GAT 394
Ile Ala Thr Leu Lys Asp Gly Arg Lys Ile Cys Leu Asp Pro Asp

GCT CCC AGA ATC AAG AAA ATT GTA CAG AAA AAA TTG GCA GGT GAT 439
Ala Pro Arg Ile Lys Lys Ile Val Gln Lys Lys Leu Ala Gly Asp

GAA TCT GCT GAT TAA TTT GTT CTG TTT CTG CCA AAC TTC TTT AAC 484
Glu Ser Ala Asp***

TCC CAG GAA GGG TAG AAT TTT GAA ACC TTG ATT TTC TAG AGT TCT 529

CAT TTA TTC AGG ATA CCT ATT CTT ACT GTA TTA AAA TTT GGA TAT 574

GTG TTT CAT TCT GTC TCA AAA ATC ACA TTT TAT TCT GAG GAA GGT 619

TGG TTA AAA GAT GGC AGA AAG AAG ATG AAA ATA AAT AAG CCT GGT 664

TTC AAC CCC TC                                             675
```
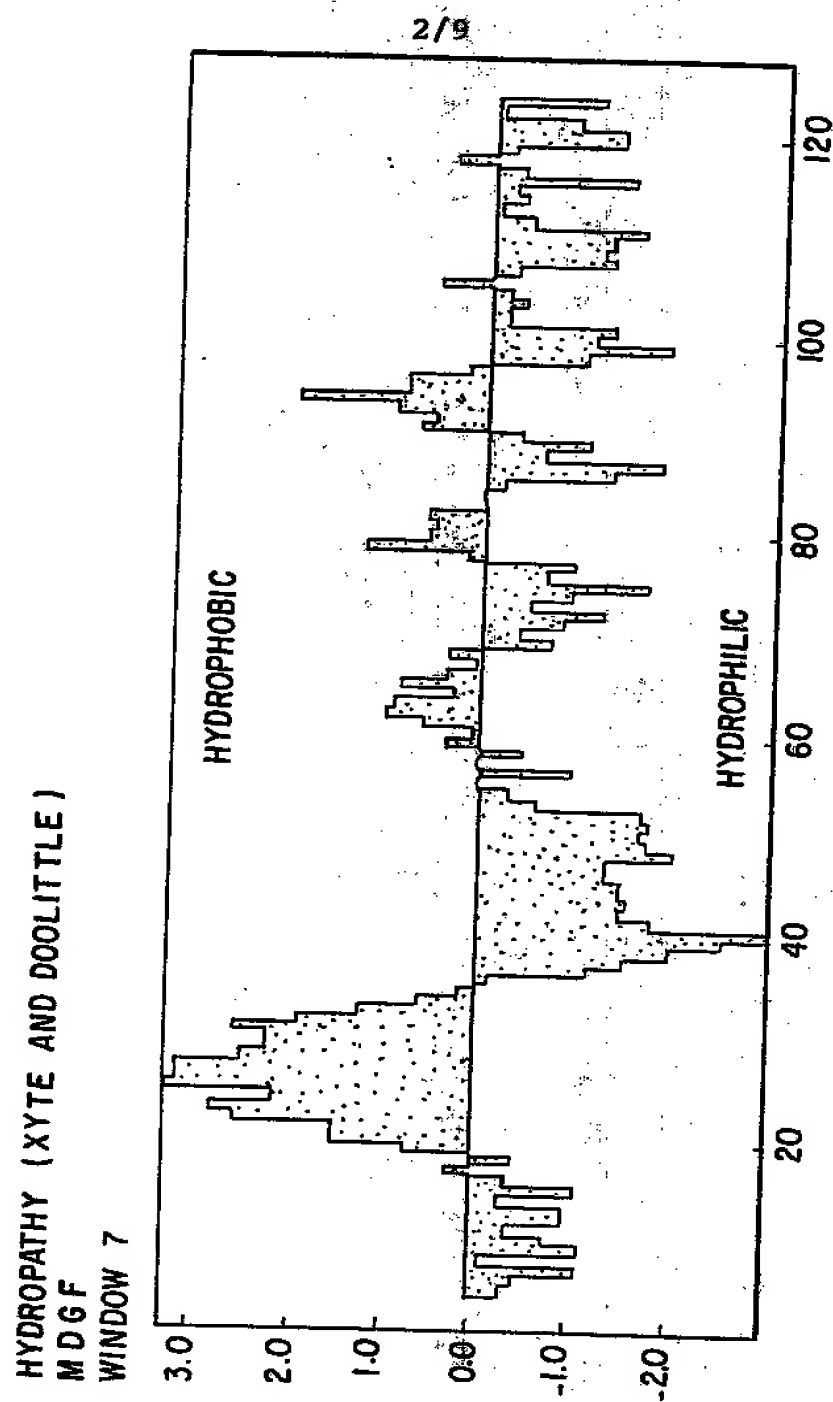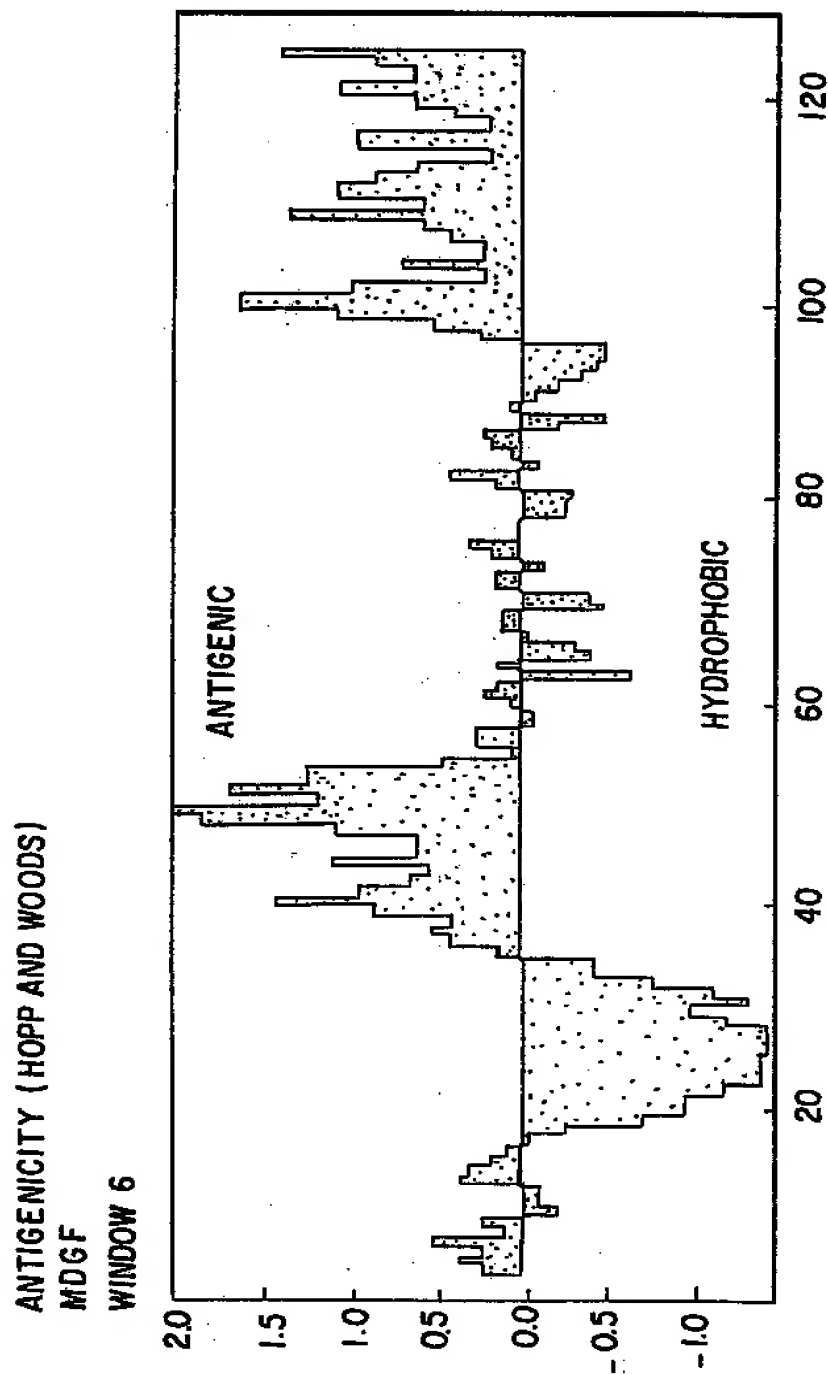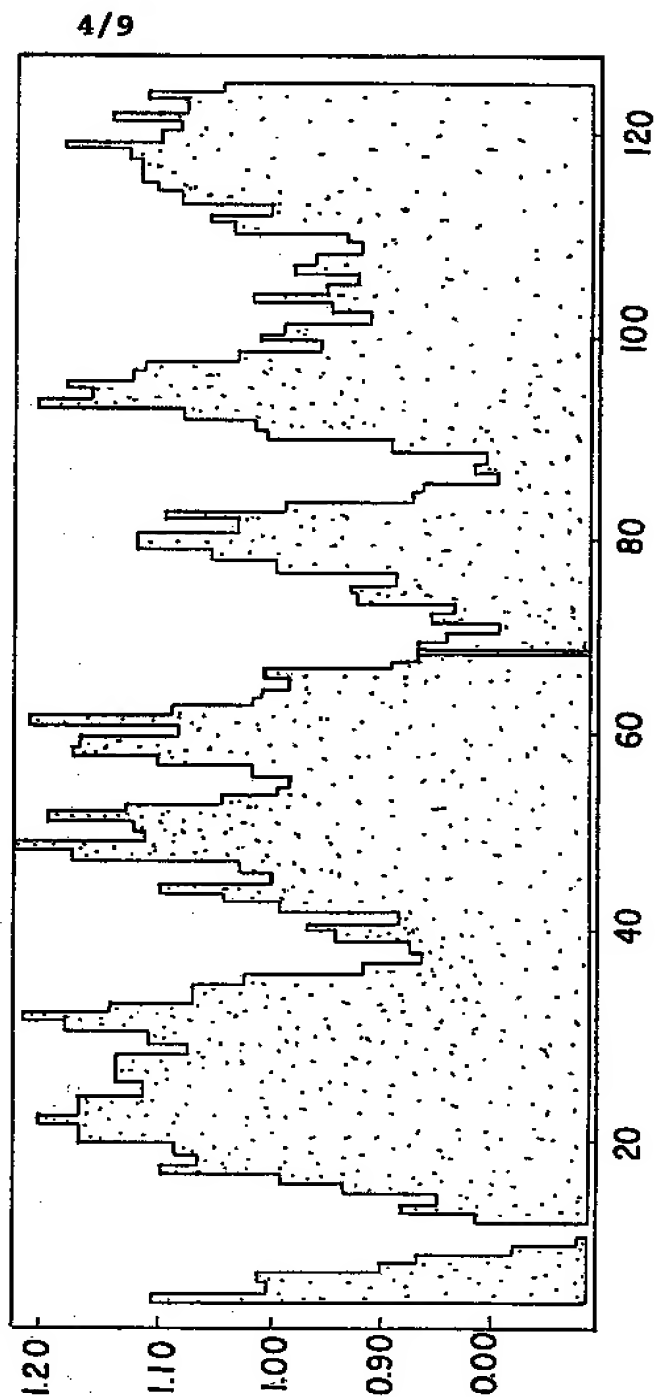
FIG. 2A.

FIG. 2B.

ANTIGENICITY (HOPP AND WOODS)
MDGF
WINDOW 6

FIG. 3A.

ALPHA HELIX POTENTIAL (CHOU AND FASMAN)
MDGF
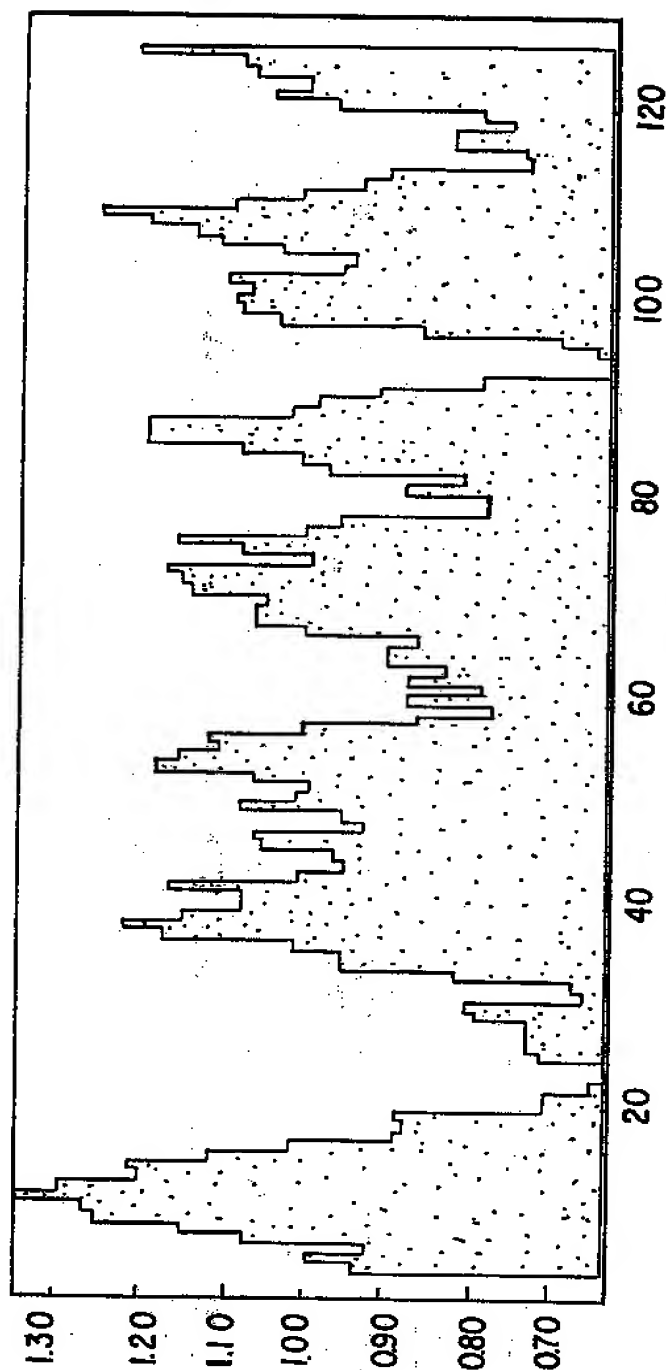WINDOW 6

5/9

F/G. 3B.

BETA SHEET POTENTIAL (CHOU AND FASMAN)
MDGF
WINDOW 6
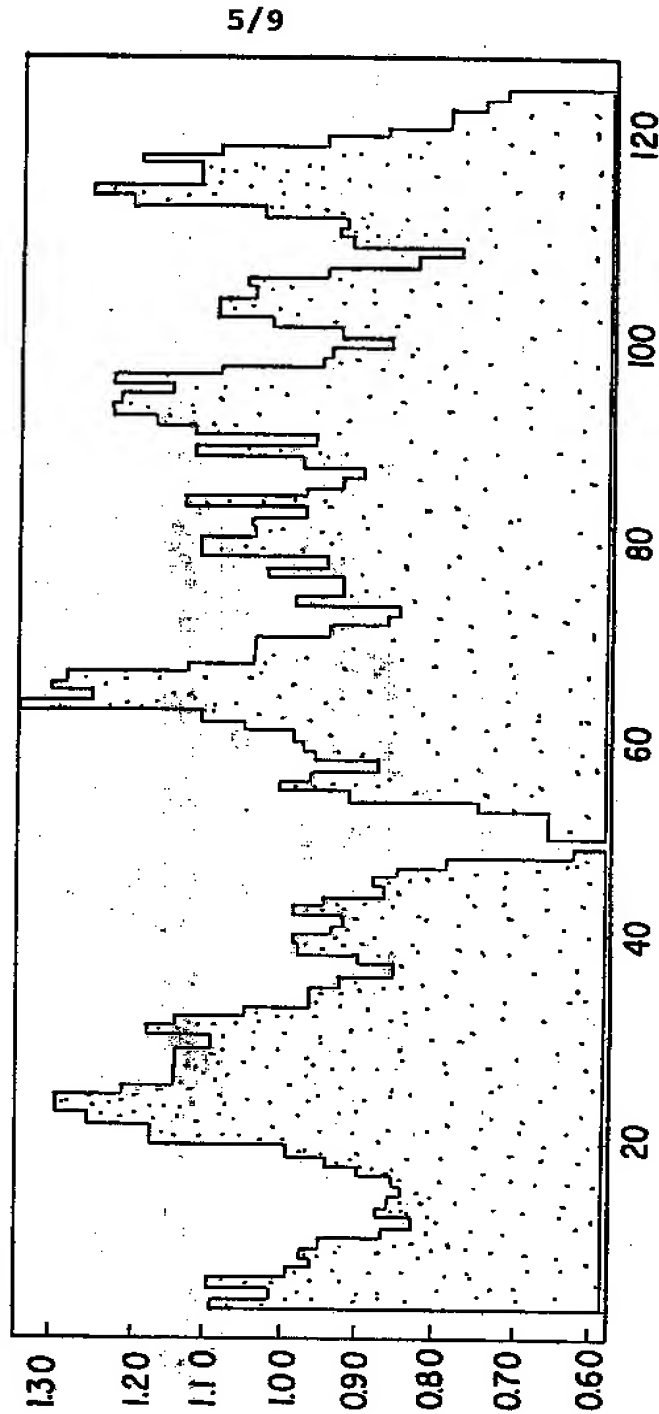
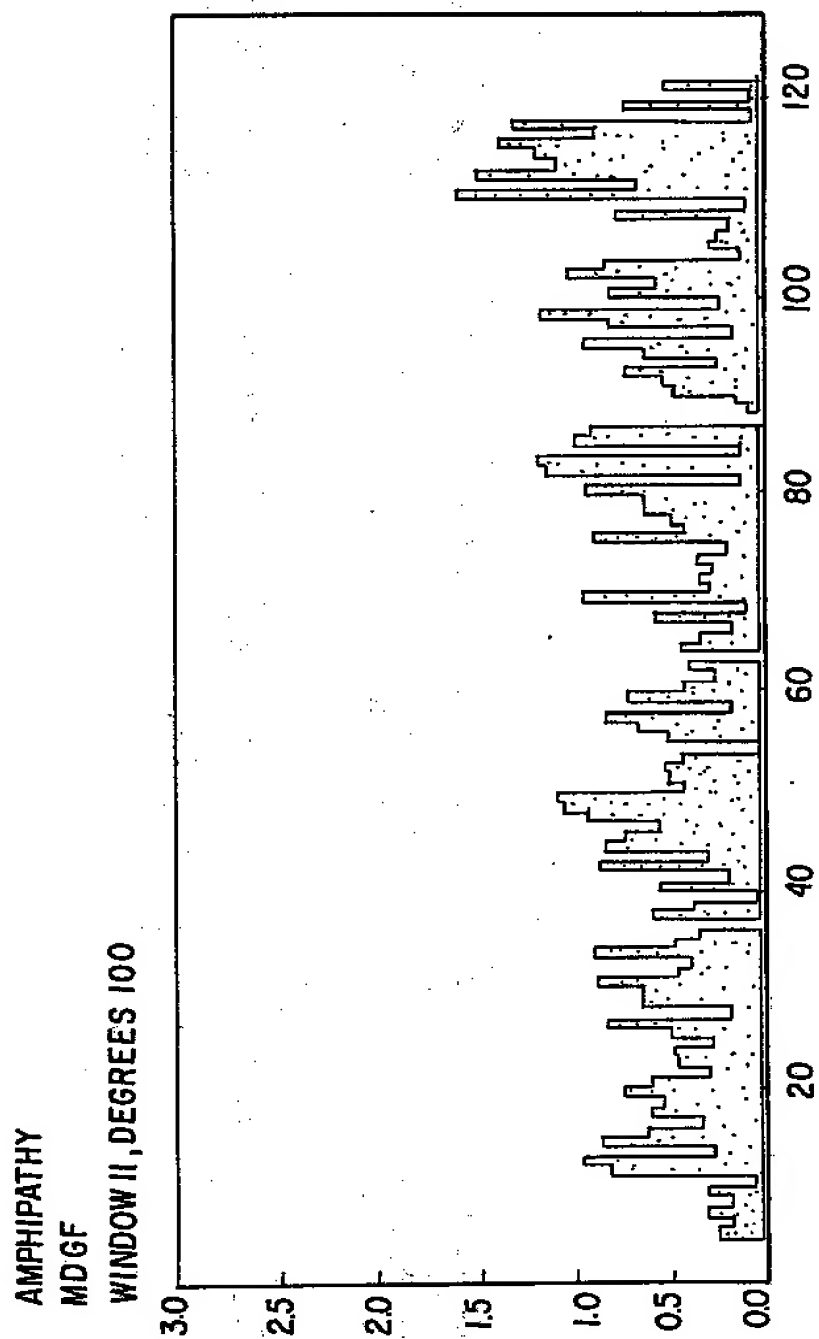FIG.3C.

BETA TURN POTENTIAL (CHOU AND FASMAN)
MDGF
WINDOW 6

7/9

FIG. 4.

NET CHARGE
MDGF
WINDOW 10

POSITIVE

NEGATIVE

FIG. 5.

AMPHIPATHY
MDGF
WINDOW II, DEGREES 100

FIG.6.